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Expression of homologous RND efflux pump genes is dependent upon AcrB expression: Implications for efflux and virulence inhibitor design

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Synopsis

Objectives Enterobacteriaceae have multiple efflux pumps which confer intrinsic resistance to antibiotics. AcrB mediates clinically relevant multi-drug resistance and is required for virulence and biofilm formation making it an attractive target for the design of inhibitors. The aim of this study was to assess the viability of single transporters as a target for efflux inhibition using *S*. Typhimurium as the model pathogen.

Methods The expression of RND efflux pump genes, in response to inactivation of single or multiple homologues was measured using real-time RT-PCR. Phenotypes of mutants were characterised by measuring antimicrobial susceptibility, dye accumulation and ability to cause infection *in vitro*.

Results Expression of all RND efflux pump genes was increased when single or multiple *acr* genes were inactivated, suggesting a feedback mechanism which activates transcription of homologous efflux pump genes. When two or three *acr* genes were inactivated, the mutants had further reduced efflux, altered susceptibility to antimicrobials (including increased susceptibility to some, but conversely and counter-intuitively, decreased susceptibility to some others) and were more attenuated in the tissue culture model than mutants lacking single pumps.

Conclusions These data indicate that it is critical to understand which pumps an inhibitor is active against and the effect of this on expression of homologous systems. For some antimicrobials, an inhibitor with activity against multiple pumps will have a greater impact on susceptibility but, an unintended consequence of this may be decreased susceptibility to other drugs, such as aminoglycosides.

Introduction

Efflux is an important mechanism of multidrug resistance in bacteria, conferring decreased susceptibility to a wide range of substrates including antibiotics, dyes, detergents and biocides.[1] This makes them an attractive target for the design of inhibitors which could be used to potentiate the use of existing antimicrobials.

Resistance nodulation division (RND) efflux transporters are found in the inner membrane of Gram-negative bacteria and form a complex with an outer membrane channel and a periplasmic adaptor protein (PAP) to form a tri-partite efflux pump system spanning both the inner and outer membrane.[2, 3] Substrates of these multi-protein complexes are structurally diverse and include antibiotics, biocides, dyes, detergents and host-derived molecules. Active efflux of substrates by RND systems is responsible for the intrinsic resistance of Gram negative bacteria to multiple classes of structurally distinct antimicrobials.[1]

Salmonella have five RND efflux pump systems: AcrAB, AcrD, AcrEF, MdtABC and MdsABC. Further RND efflux pumps are found in some other members of the Enterobacteriaceae including MdtF in *Escherichia coli.*[4] The transporter protein, AcrB, and its homologues in other Gram-negative bacteria, is considered the most clinically relevant RND system because it has the broadest substrate profile and is more abundant within the cell than other efflux systems.[1] Inactivation of *acrB* increases susceptibility of laboratory mutants of *E. coli*, *Salmonella enterica* and other Enterobacteriaceae to many antimicrobials, whereas overexpression confers multi-drug resistance (MDR), including to clinically efficacious agents. Such mutants have been selected *in vitro* and *in vivo.*[5-8] Efflux via AcrB is driven by the proton-motive force and forms a tri-partite complex with the PAP, AcrA, and the outer

membrane channel, TolC.[9] The recent elucidation of the structure of AcrB in complex with different substrates of varying molecular weights has revealed two large, discrete, multisite binding pockets within AcrB, which may explain how AcrB can transport such structurally varied substrates. [10, 11]

Single deletions of RND efflux pump genes other than *acrB* have little or no effect on the susceptibility of Enterobacteriaceae to most antimicrobial agents.[12] Antimicrobial susceptibility of deletion mutants and strains with increased expression of certain RND efflux pumps indicates that there is overlap, or redundancy between the antimicrobials, biocides, dyes and detergents that can be transported by the different RND pumps of *Salmonella.*[12, 13] AcrF is the closest homologue of AcrB in *Salmonella* (80% identity) and AcrEF overexpression can suppress antibiotic hyper-susceptibility in AcrB deficient strains.[12, 13] AcrD (64% identity to AcrB) and MdtABC have similar substrate profiles including SDS, novobiocin, deoxycholate, some β-lactams, copper and zinc.[12, 14] In *E. coli* and *Salmonella,* AcrD also transports aminoglycoside antibiotics.[15, 16] MdsABC is found only in *Salmonella* and in LB medium is expressed at lower levels than the other four RND efflux pumps.[17] However, overexpression of *mdsAB*, encoding the pump and periplasmic adaptor protein, or *MdsABC*, encoding all three components, were overexpressed in a strain lacking AcrB, susceptibility to novobiocin, acriflavine, crystal violet, methylene blue, rhodamine 6G, benzalkonium chloride and SDS was decreased compared to the mutant suggesting that MdsB is capable of exporting these compounds.[18]

In addition to the role in resistance to antimicrobials, some RND efflux pumps are also required for virulence of many Gram-negative pathogens.[19] In *S.* Typhimurium, inactivation of *acrB* attenuated invasion of tissue culture cells *in vitro* and colonisation in poultry.[20, 21]

89 Inactivation of *acrAB* or *acrEF* has also been shown to attenuate the virulence of the
90 organism in mice.[12]

91 Regulation of RND efflux pumps is complex; transcriptional control is multi-layered and
92 some regulators control expression of more than one pump. In *Salmonella* and *E. coli*, the
93 regulation of *acrAB* is the best studied. The *acrA* and *acrB* genes are encoded in a single
94 operon and are co-regulated. At a local level *acrAB* is repressed by AcrR, which is encoded
95 alongside and divergently transcribed from *acrAB*. At a global level members of the
96 AraC/XylS family of DNA transcriptional activators, such as MarA, SoxS, Rob and RamA, all
97 influence expression of *acrAB-tolC* and have overlapping recognition sites.[22-27] AcrD and
98 MdtABC are both under the control of the two-component regulatory systems BaeSR and
99 CpxAR which induce expression of AcrD and MdtABC in response to high levels of indole,
100 copper, zinc or envelope stress.[28, 29] Expression of *acrEF* in *E. coli* is generally low due to
101 repression by the global regulator H-NS.[30] However, *acrEF* is also encoded alongside a gene
102 encoding a local repressor, AcrS (previously EnvR) which inhibits expression of *acrAB* and
103 acts as a regulatory switch between expression of *acrAB* and *acrEF.*[31]

104 Due to the functional redundancy of RND pumps, potential exists for the loss of certain
105 pump components to be compensated by increased expression of a homologous
106 component that could fulfil, at least to some extent, the same function. For example, Eaves
107 and colleagues [32] showed that when *acrB* or *acrF* of *Salmonella* was inactivated, expression
108 of *acrD* increased (3.6 and 4.9 fold, respectively). To allow compensatory changes in
109 expression levels of efflux systems upon inactivation of homologous systems, there must be
110 a tightly controlled and integrated regulatory network that can respond to loss of efflux
111 function. Whilst the literature contains multiple examples of regulation of single efflux

112 systems or a small number of efflux systems, an integrated network of regulation is yet to
113 be elucidated.

114 The role of RND systems in both antimicrobial resistance and virulence makes them
115 attractive targets for the design of inhibitors. Using *S*. Typhimurium as a model, the aim of
116 the present study was to investigate the viability of single transporters such as AcrB as a
117 target for efflux inhibition by investigating the expression and roles of structurally similar
118 efflux pumps in antimicrobial resistance and virulence, and the extent of the redundancy
119 between RND efflux pumps. This information is crucial for the rational design of inhibitors
120 which inhibit all pumps so preventing resistance by compensatory over-production of
121 homologous RND efflux systems.

Materials and Methods

Strains and growth conditions. All strains were derived from *Salmonella enterica* serovar Typhimurium SL1344.[33] *Salmonella* was used as a model organism in this study as it is an important human pathogen that causes a significant number of infections annually. SL1344 is a widely studied pathogenic strain of *Salmonella* for which there are well validated models of infection including an *in vitro* tissue culture model. Single gene inactivated mutants were constructed as described previously.[32, 34] Mutants with multiple efflux pumps inactivated were created by P22 transduction between mutants in which single genes were inactivated or deleted. All mutants were verified by PCR and DNA sequencing. All experiments including MICs reveal that the phenotype of the marked and unmarked mutants for the same gene is indistinguishable. LB broth (Sigma-Aldrich, UK) and MOPS minimal medium (Teknova Inc., USA) were used throughout this study.

RNA extraction and Real Time qRT-PCR. Overnight cultures of *S*. Typhimurium SL1344 and the test strains were grown in MOPS minimal medium at 37°C. From each strain three biological replicate RNA preparations were made and quantified as described previously.[22, 35] Primers (Supplementary Table 1) were designed with an annealing temperature of 57.3˚C using Beacon Designer 4.0 (Premier Biosoft, USA). cDNA was synthesised from 2 µg total RNA using superscript III cDNA synthesis kit (Invitrogen). Validation experiments were carried out using 5 cDNA standards of different concentrations (10, 1, 0.1, 0.01, 0.001 ng/µl) to determine PCR efficiency for the housekeeping gene 16S and each test gene. qRT-PCRs were set up in biological triplicate and technical duplicate in a BIORAD PCR tray using 1 µl neat cDNA for test genes and 1 µl of a 1:1000 dilution cDNA for 16S in a 25 µl reaction containing 12.5 µl iQ SYBR green supermix (BIORAD, UK), 1 µl primers (500nm) and 9.5 µl

sterile water. qRT-PCR was carried out in a CFX-96 Real-time machine (BIORAD, UK) using the following protocol; 95˚C for 5 minutes followed by 40 plate read cycles of 95˚C for 30 s, 57.3˚C for 30 s and 72˚C for 30 s. Data were analysed using CFX Manager (BIORAD, UK) and expression ratios were calculated using the ΔΔct method and normalised to expression of 16S.[36]

Determination of susceptibility to antimicrobials. Biolog data was confirmed by measuring growth in the presence of representative AcrB substrates. Briefly, overnight bacterial cultures were diluted to 10^4 CFU/ml and grown in a 96 well plate in iso-sensitest broth in the presence of selected drugs at 0.25 x the wild-type MIC.

The minimum inhibitory concentrations (MIC) of antibiotics, dyes and detergents were determined for each strain according to the standardised agar doubling dilution method procedure of the British Society of Antimicrobial Chemotherapy (BSAC) using iso-sensitest agar.[37] The MIC was determined as the lowest concentration of antimicrobial that caused no visible growth. Values stated are the mode value from at least three biological replicates performed on at least three independent occasions. All antimicrobials tested were obtained from Sigma, UK.

Accumulation of Hoechst 33342 and norfloxacin. The efflux activity of the mutants was assessed by determining the accumulation of the fluorescent dye Hoechst 33342 and norfloxacin (Sigma, UK) as described previously.[38, 39] Differences in steady state accumulation values between mutants and parental strains were analysed for statistical significance using a two-tailed Student's t-test, where $P < 0.05$ was considered significant. Data presented are the mean of three independent biological replicates ± standard error of the mean.

Adhesion and invasion assays. The ability of the strains to adhere to, and invade, INT-407 (human embryonic intestine cells) was measured as previously described.[21] Each assay was repeated a minimum of three times, with each experiment including four technical replicates per bacterial strain. The results were analysed using Student's *t*-test and P values of ≤ 0.05 were considered significant.

Results

In silico analysis

The *Salmonella* genome encodes five efflux systems of the RND family. The three most similar pump proteins are AcrB, AcrD and AcrF (Table 1). The two remaining RND pumps, MdsB and MdtB/C, which is a heteromultimer of MdtB and MdtC, are 46.7% and 48.6% identical to AcrB, respectively. ClustalW alignments of all six RND type proteins revealed that five residues (Asp407, Asp408, Lys940, Arg971 and Thr978) vital to proton transport within AcrB of *E. coli* [40, 41] are identical in all six *Salmonella* proteins indicating that energy transduction is conserved in this family of proteins. However, greater variation was seen in residues involved in substrate recognition or binding. For example, of six important residues in the hydrophobic, phenylalanine rich binding pocket of AcrB (Phe178, 615, 610, 136, 617 and 628) [42] all six were conserved in AcrF, two in AcrD, one in MdtC, one in MdsB and none of these residues were found in MdtB.

Expression of efflux pump genes is altered upon inactivation of homologous pumps.

To determine whether the expression of each gene was altered upon inactivation of one or more homologous genes, real-time quantitative RT-PCR was used to measure the level of *acrB*, *acrD* and *acrF* transcription in the single, double and triple efflux mutants compared to SL1344 (Table 2). As shown previously, in the *acrB* mutant both *acrD* and *acrF* had increased expression.[32] In the *acrD* mutant, expression of *acrB* was increased while *acrF* expression was unchanged. In the *acrF* mutant both *acrB* and *acrD* had increased expression. The expression of *mdsB* and *mdtB,* was also affected by loss of single RND pumps. The expression of *mdtB* was increased in the *acrF* mutant while expression of the *Salmonella* specific efflux pump *mdsB* was decreased upon inactivation of *acrB*, *acrD* or *acrF* (Table 2).

When two genes were inactivated (e.g. *acrB* and *acrF* or *acrB* and *acrD*) expression of *acrD* or *acrF* was increased, although expression was lower than in the strain lacking only *acrB* (L110). When *acrF* and *acrD* were inactivated expression of *acrB* was increased, this was greater than that seen upon inactivation of *acrD* and similar to that in the *acrF* mutant. Expression of the *mdtB* and *mdsB* efflux genes was increased in all *acr* gene double mutants (L646, L1297 and L1395) but in the triple *acrBDF* mutant only *mdtB* expression was increased. Expression of both *mdtB* and *mdsB* was highest in the *acrDF* mutant (L1395) (Table 2).

The expression level of known regulators of RND efflux was also measured. The expression of *ramA* and *marA* was increased when *acrB* was inactivated but not changed in the *acrD* (L132) or *acrF* (L131) mutants (Table 2). Expression of *soxS* was increased in the *acrBF* mutant (6.3 fold), in the *acrDF* mutant (2.2 fold) and in the strain lacking all three RND pump genes. Expression of *rob* was not significantly altered in any of the mutants. The expression of the genes encoding the repressor proteins AcrR and EnvR/AcrS was also measured. Transcription of *acrR* was decreased in the *acrB* mutant and transcription of *envR* was increased in the *acrBDF* mutant (Table 2).

Inactivation of two or more RND efflux systems altered antimicrobial susceptibility.

As previously described, inactivation of *acrB* led to multi-drug hyper-susceptibility while single inactivation of either *acrD* or *acrF* did not significantly alter MICs of antibiotics, dyes and detergents compared to the wildtype strain. Re-interrogation of previously published data [43] from the Biolog Phenotype Microarray showed that the *acrD* and *acrF* mutants grew better than SL1344 when exposed to four β-lactams, five macrolides, and five quinolones

(Supplementary Table S2). This observation was confirmed by measuring the growth kinetics of the strains in the presence of representative AcrB substrates. However, the beneficial effect of lacking *acrD* or *acrF* was lost when *acrB* was deleted in the same strain (L1297 and L646, respectively) (Figure 1).

The antimicrobial susceptibility of the double mutant lacking AcrD and AcrF (L1395) was not significantly different from that of SL1344 (Table 3). Furthermore, except for ethidium bromide (for which the MIC value was lower) and the aminoglycosides (for which the MIC values were increased), the susceptibility of the *acrBD* (L1297), *acrBF* (L646) and the triple *acrBDF* (L1405) mutants to antibiotics, dyes and detergents was not significantly different to that of the *acrB* mutant. Surprisingly, the MICs of the aminoglycoside antibiotics, streptomycin, gentamicin and amikacin, were higher for the *acrBF* (L646) mutant and the triple *acrBDF* mutant (L1405) than for the wild-type parental strain SL1344; the MIC of tobramycin was also greater for L1405 than SL1344 (Table 3B). All *acrB* mutants (L110, L646, L1297 and L1405) were more susceptible to the efflux inhibitors Phe-arg-β-naphthylamide (PAβN) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) than the wild-type parental strain (SL1344).

Inactivation of two or more RND efflux systems decreased efflux activity.

We previously showed that inactivation of *acrB* led to increased accumulation of the dye Hoechst 33342.[20] Compared to SL1344, inactivation of *acrD* (L132) or *acrF* (L131) or inactivation of both *acrD* and *acrF* (L1395) did not significantly alter the accumulation of Hoechst 33342 (Figure 2). However, mutants with *acrB* and another *acr* gene inactivated (*acrBD* (L1297) or *acrBF* (L646)) accumulated less Hoechst 33342 than the *acrB* mutant. The *acrBDF* mutant (L1405) accumulated the highest level of Hoechst 33342 indicating the

lowest level of efflux. Accumulation of the fluoroquinolone antibiotic, norfloxacin, showed a similar pattern although the *acrB* mutant accumulated a higher concentration than the *acrBDF* mutant (Figure 3).

Inactivation of two or more RND efflux systems attenuated the ability of *Salmonella* to infect tissue culture cells.

In addition to their role in antimicrobial resistance, in many Gram-negative bacterial pathogens RND efflux pumps are required for the ability to cause infection.[19] Inactivation of *acrB* has been previously shown to attenuate invasion of *S*. Typhimurium into mammalian cells growing in tissue culture.[12, 20, 21] We now show that single gene inactivation of either *acrD* (L132) or *acrF* (L131) also significantly attenuate the virulence of *Salmonella*. Adhesion by L131 (*acrF::aph*) to human intestinal cells (INT-407) was 56.0% that of SL1344 and it only invaded at 39.1% of wild-type level. L132 (*acrD::aph*) was even more attenuated; adhesion was only 29.0% that of SL1344 and invasion was only 39.1% of the SL1344 level (Figure 4).

When two efflux pump genes were inactivated or deleted, the ability of *Salmonella* to adhere to, and invade, INT-407 cells was attenuated more than that seen in single *acr* mutants (Figure 4). Invasion of the *acrBD* double mutant was the lowest among all single and double mutants at only 0.4% of wild-type level. The adhesion of the *acrDF* mutant, L1395 (*acrD::cat, acrF::aph*), was 83.7% that of SL1344 adhesion, which is significantly greater than mutants lacking only one of these systems. Despite this, the invasion level of this mutant was only 17.8% that of the parental strain showing that these two mutations have an additive effect upon invasive ability. When all three of the efflux genes were inactivated in L1405, *Salmonella* was almost completely unable to adhere to or invade INT-407 cells (adhesion = 0.16% of wild-type level, invasion = 0.004% of wild-type level).

Discussion

The role of RND efflux pumps in multi-drug resistance and virulence makes them attractive targets for the design of efflux inhibitors. We have shown that expression of all RND efflux pump genes can be altered when single or multiple *acr* genes are inactivated. These data suggest that the bacterium can sense and respond to the levels of RND transporters and due to overlapping substrate specificity this affords resilience to the bacterium to prevent intracellular accumulation of toxic metabolites,[44-47] or survival in toxic environments.

Critically, we can correlate alteration in efflux level and sensitivity to antimicrobials to the compensatory changes in efflux pump gene expression in strains lacking specific RND pumps. For example, in the Biolog phenotype microarray many of the compounds in which the *acrD* and *acrF* mutant grew better are known substrates of AcrB which is over-expressed in these mutants. Furthermore, this beneficial effect is lost when *acrB* is also inactivated. Other than to the aminoglycosides, the susceptibility of the *acrBD* and *acrBF* mutants was not significantly different to that of the mutant lacking only *acrB* and it is likely that this is because the other three RND systems are overproduced in both cases.

AcrD, which is known to transport aminoglycosides, and MdtB, which has a similar substrate profile to AcrD, are over-expressed in the *acrBF* mutant. This could explain the decreased susceptibility to the aminoglycosides seen in this mutant. The *acrBDF* mutant also had decreased susceptibility to the aminoglycosides. Expression of *mdsB* is increased in this mutant but there is currently no evidence this pump can transport aminoglycosides. Aminoglycosides enter bacterial cells by self-promoted uptake and it is possible that changes in expression of genes encoding cell envelope components, including LPS, could be responsible for this effect.[48]

These data provide proof of principle that changes in expression of pumps in response to inactivation of RND efflux pumps can alter susceptibility to clinically relevant antimicrobials. We postulate the same will be true when the pump proteins themselves are inhibited and recent evidence showing that the efflux inhibitors PAβN and NMP altered expression of RND efflux pump genes in *E. coli* supports this.[49] Additionally, this highlights that any change in the phenotype of strains with single or multiple genes inactivated should be interpreted with caution as the phenotype represents, the engineered inactivation and any consequent transcriptional changes.

The role of AcrAB-TolC in virulence of *S*. Typhimurium is well established and inactivation of *acrB* causes decreased expression of genes in SPI-1, which are known to be required for infection.[12, 20, 21, 35] Nishino *et al*., showed that inactivation of *acrD* did not confer significant attenuation in the BALB/C mouse model of infection and inactivation of *acrEF* (encoding the RND pump protein and the periplasmic adaptor protein) increased the host survival rate with 20% of mice surviving to 21 days rather than none when infected with the wild-type strain.[12] In the tissue culture model lack of either AcrD or AcrF caused a significant reduction in the ability of *Salmonella* to infect INT-407 cells with the *acrD* mutant (L132) being more attenuated than the *acrF* mutant (L131). There are several hypotheses to explain these data. First, as inactivation of *acrB* is known to alter expression of genes found in SPI-1 [35] it is possible that inactivation of other RND pump genes also affects virulence gene expression. Alternative explanations include that RND efflux pumps export substrates that are required for infection or that absence of some RND efflux pumps causes damage or stress to the bacterial cell membrane which compromises the ability to cause infection.

311 The effect of inactivating *acrB* plus one or two other efflux pump genes upon the ability to
312 cause infection was additive. The *acrDF* (L1395) mutant was less attenuated than either of
313 the single mutants (L131 and L132). One explanation for this is that *acrB, mdtB* and *mdsB*
314 are all overexpressed in this mutant and so are able to partially compensate for the
315 functions of the other two systems. The triple mutant lacking AcrB, AcrD and AcrF was
316 unable to adhere to, or invade the INT-407 cells. This could suggest that no other
317 transporter could compensate for the loss of these proteins or that inactivation of multiple
318 RND efflux pump genes causes greater changes in expression of virulence genes.

319 The role of efflux pumps in antibiotic resistance makes them targets for the design of
320 inhibitors. Due to the role of efflux pumps in virulence we also postulate that efflux
321 inhibitors will inhibit virulence as well as augment the activity of antibacterial drugs. Our
322 data show that inhibitors designed to inhibit all RND efflux systems will have a greater anti-
323 virulence effect on the organism.

324 The compensatory expression of efflux pump genes was associated with changes in
325 regulatory gene expression. We hypothesise that the bacterial cell is attempting to increase
326 expression of the inactivated/deleted genes by increasing expression of factors known to
327 regulate expression of RND efflux pump genes such as *ramA*, *marA*, *soxS* and *rob.*[22-24, 26, 27,
328 43, 50] Our data suggest that these regulators are involved in the modulation of RND efflux
329 pump expression in the absence of homologous systems. Expression of *ramA* was increased
330 when *acrB* was inactivated,[51] however, expression of *soxS* was increased when two or more
331 *acr* genes were inactivated. SoxS is also a transcription factor of the AraC/XylS family
332 involved in regulating the response to oxidative stress and genes including *acrAB* and *micF.*[52]
333 Increased expression of *soxS* could suggest that lack of efflux by Acr pump proteins leads to

accumulation of toxic metabolites, as proposed by Rosner and Martin when *E. coli tolC* is inactivated.[44, 47]

The critical role of RND systems in both antimicrobial resistance and virulence of pathogenic bacteria makes them attractive targets for the design of inhibitors. These molecules could be used to re-sensitise the bacterium to antimicrobials whilst simultaneously attenuating virulence of the infecting organism. Critically, our data indicate that care should be taken when developing efflux pump inhibitors against the RND pumps to determine which pumps are inhibited and to understand the effect of this on expression of homologous systems. In terms of attenuating virulence, the effect of inhibition was additive so inhibition of multiple pumps is a good strategy. However, the benefit of this strategy on increasing susceptibility to antimicrobials may be more complex and the impact of this will depend on which drugs are used to treat infections by a particular pathogen. For some antimicrobials, an inhibitor with activity against multiple pumps will have a greater impact on susceptibility but, an unintended consequence of this may be decreased susceptibility to other drugs, such as the aminoglycosides.

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Transparency declaration

The authors declare no conflict of interest.

358 **Figure legends**

359 **Figure 1. Growth of efflux pump mutants in the presence of AcrB substrates.** Growth of the
360 efflux in the presence of (a) ciprofloxacin and (b) doxycycline at a concentration of 0.25 x
361 the MIC for wild-type. Data presented is the mean of three biological replicates.

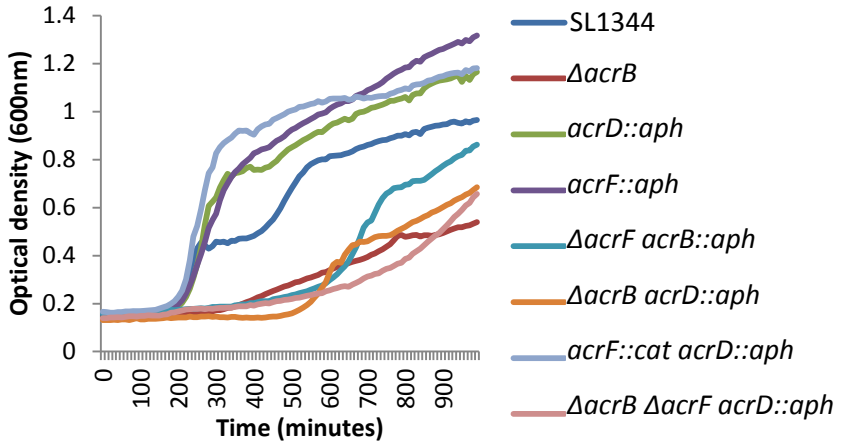
362 **Figure 2. Hoechst 33342 Accumulation in (A) single efflux mutants and (B) double and**
363 **triple efflux mutants.** The data presented are the mean of three separate experiments
364 presented as fold change compared to SL1344 at the end point of the assay ± standard error
365 of the mean. Student's t-tests were performed to compare the Hoechst 33342 accumulation
366 of each strain to that of SL1344 and those returning *P* values of less than 0.05 are indicated
367 by *.

368 **Figure 3. Accumulation of norfloxacin.** Data shown is the mean of three biological replicates
369 +/- the standard error of the mean.

370 **Figure 4. Adhesion (A) and Invasion (B) of strains lacking AcrB, AcrD, AcrF and**
371 **combinations thereof to invade INT-407 cells *in vitro*.** Data shown is the mean of at least
372 three independent experiments. Student's *t*-tests were used to compare values for each
373 strain with that of the wild-type, SL1344. P values of ≤ 0.05 were considered significant and
374 are indicated by asterisks.

375

376 Figure 1.



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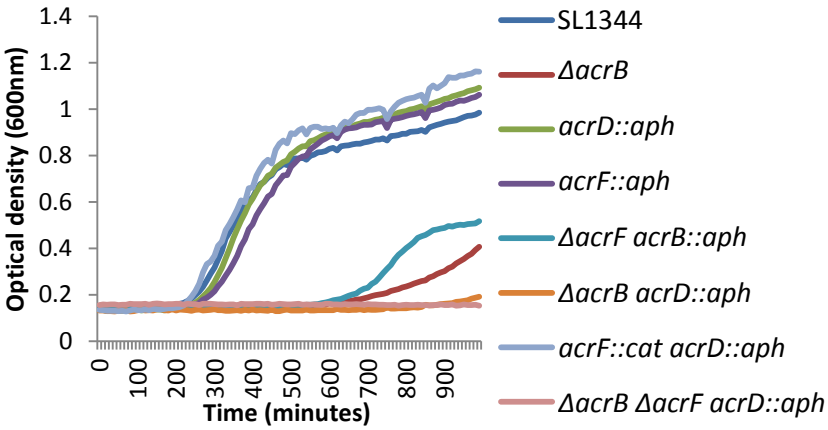

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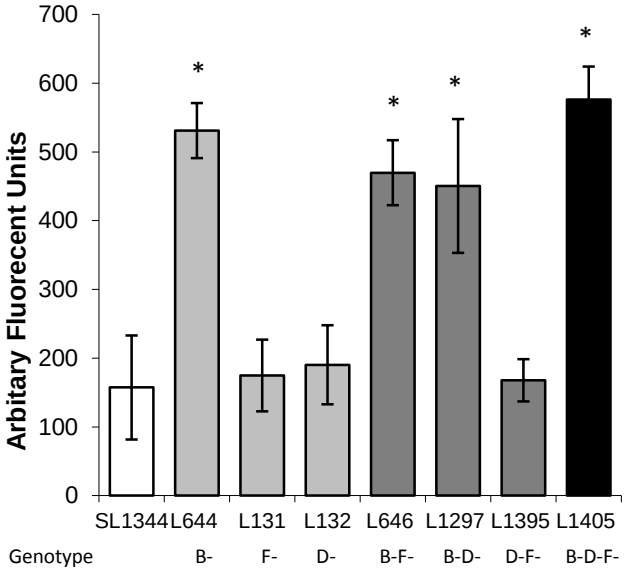
379 **Figure 2. Hoechst 33342 accumulation**



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382 **Figure 3. Accumulation of norfloxacin**

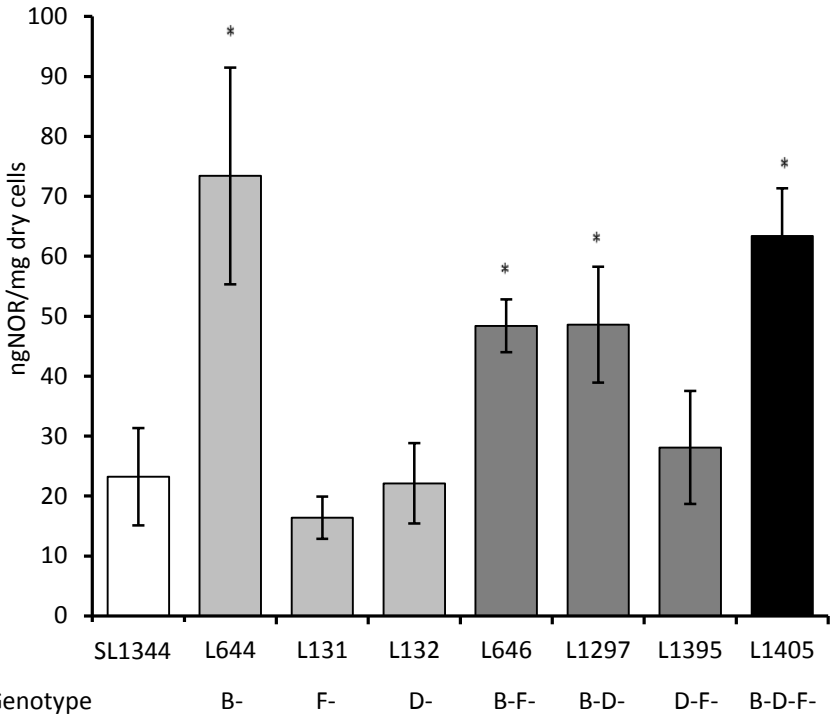

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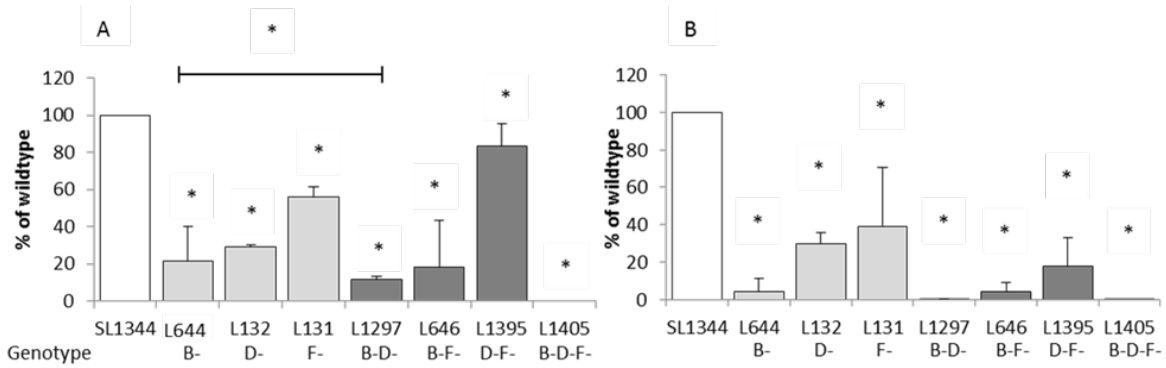
Figure 4. Adhesion (A) and Invasion (B) of strains lacking AcrB, AcrD, AcrF and combinations thereof to invade INT-407 cells *in vitro*



389 **Table 1. Percentage nucleotide identity and amino acid similarity between RND efflux**

390 **pump genes and proteins of *Salmonella***

	acrB / AcrB	*acrD* / AcrD	*acrF* / AcrF	*mdtB* / MdtB	*mdtC* / MdtC	*mdsB* / MdsB
acrB/AcrB	-	70 / 79.1	74 / 90.4	55 / 46.7	55 / 48.6	59 / 63.4
acrD/AcrD	-	-	68 / 78.2	54 / 49.1	54 / 48.8	59 / 61.3
acrF/AcrF	-	-	-	54 / 47.4	53 / 48.5	57 / 63.1
mdtB/MdtB	-	-	-	-	62 / 66.0	56 / 49.0
mdtC/MdtC	-	-	-	-	-	56 / 49.2
mdsB/MdsB	-	-	-	-	-	-

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Table 2. Expression of RND efflux pump genes and regulators thereof, quantified by real time RT-PCR

		Fold change in gene expression										
		RND efflux pump genes					Known regulators of efflux					
Strain		*acrB*	*acrD*	*acrF*	*mdtB*	*mdsB*	*marA*	*ramA*	*rob*	*soxS*	*acrR*	*envR*
SL1344	WT	1.0	1.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
L644	*ΔacrB*	-	**13.4**	**16.0**	1.0	*0.6*	**2.0**	**2.8**	1.4	**2.6**	**0.4**	1.2
L132	*acrD::aph*	**1.8**	-	1.0	0.9	*0.5*	1.2	0.8	1.4	1.3	0.3	0.6
L131	*acrF::aph*	**3.4**	**3.4**	-	**1.8**	*0.7*	0.9	1.0	1.3	1.4	0.9	1.5
L646	*ΔacrF acrB::aph*	-	**4.6**		**4.5**	**3.0**	**2.5**	1.6	1.2	**6.3**	0.4	0.8
L1297	*ΔacrB acrD::aph*	-	-	**2.4**	**4.6**	**2.1**	1.2	1.4	1.1	1.8	2.2	1.2
L1395	*ΔacrF acrD::aph*	**3.8**	-	-	**6.0**	**5.9**	1.0	1.3	1.4	**2.2**	0.4	1.0
L1405	*ΔacrB ΔacrF acrD::aph*	-	-	-	1.1	**3.2**	1.6	1.9	1.0	**5.0**	0.5	**4.5**

Bold text indicated statistically significant ($P \leq 0.05$) increased expression. Italic text indicates statistically significant decreased expression.

Table 3. Antimicrobial susceptibility of SL1344 and RND mutants thereof.

A. Minimum Inhibitory Concentration of antimicrobials to SL1344 and mutants thereof.

		MIC (mg/L)													
	Genotype	Amp	Chl	Cip	Tet	Nal	EtBr	Fus	Ami	Gent	Hyg	Strep	Tob	PAβN	CCCP
SL1344	Wild-type	2	4	0.015	1	4	>256	>256	4	0.5	32	8	2	>1024	64
L110	*acrB::aph*	*0.25*	*1*	<0.008	0.5	*1*	*64*	*8*	4	1	32	8	1	*64*	32
L644	*ΔacrB*	*0.25*	*1*	<0.008	0.5	*1*	*64*	*8*	4	1	32	8	1	*64*	32
L131	*acrF::aph*	2	4	0.015	2	4	>256	>256	4	1	32	16	2	>1024	64
L132	*acrD::aph*	2	4	0.015	1	4	>256	>256	4	1	32	16	2	>1024	64
L646	*ΔacrF acrB::aph*	2	*1*	<0.008	1	*1*	*16*	*4*	8	**2**	64	**32**	4	*64*	32
L1297	*ΔacrB acrD::aph*	*0.25*	*1*	<0.008	0.5	*1*	*64*	*8*	2	0.5	32	8	1	*64*	32
L1395	*acrF::cat acrD::aph*	2	16	0.015	2	4	>256	>256	4	1	64	16	2	>1024	64
L1405	*ΔacrB ΔacrF acrD::aph*	*0.12*	*1*	<0.008	0.5	*1*	*16*	*8*	**16**	**2**	64	**32**	**8**	*64*	32

B. Fold change in MIC compared to Δ*acrB* (L644)

	Genotype	Amp	Chl	Cip	Tet	Nal	EtBr	Fus	Ami	Gent	Hyg	Strep	Tob	PAβN	CCCP
L646	*ΔacrF acrB::aph*	**4**			**2**		*-4*	*-2*	**2**	**2**	**2**	**4**	**4**		
L1297	*ΔacrB acrD::aph*								*-2*	*-2*	*-2*				
L1405	*ΔacrB ΔacrF acrD::aph*	*-2*					*-4*		**4**	**2**	**2**	**4**	**8**		

Amp, ampicillin; Chl, chloramphenicol; Tet, tetracycline; Nal, nalidixic acid; EtBr, Ethidium bromide; Fus, Fusidic Acid; Ami, Amikacin, Gent, Gentamicin; Hyg, Hygromycin; Strep, Streptomycin; Tob, Tobramycin; PAβN, Phe-Arg β-naphthylamide dihydrochloride; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone. The *aph* gene used is *aph*(3')-1 which gives resistance to kanamycin, neomycin and paromycin.

Table 3A: Bold red font indicates an increase in the MIC of the same compound compared to SL1344. Italic blue font indicates a decrease in the MIC of the same compound compared to SL1344. Table 3B: Bold red font indicates an increase in MIC compared to the same compound for L644. Italic blue font indicates a decrease in the MIC of the same compound for Δ*acrB*. No value indicates no difference in MIC values.

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